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Iron-sulphur cluster nitrosyls, a novel class of nitric oxide generator: mechanism of vasodilator action on rat isolated tail artery

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1 Two iron-sulphur cluster nitrosyls have been investigated as potential nitric oxide (NO·) donor drugs (A: tetranitrosyltetra-μ3-sulphidotetrahedro-tetrairon; and B: heptanitrosyltri-μ3-thioxotetraferrate(1-)). Both compounds are shown to dilate precontracted, internally-perfused rat tail arteries.

2 Bolus injections (10 μl) of compound A or B generate two kinds of vasodilator response. Doses below a critical threshold concentration (D_T) evoke transient (or *T*-type) responses, which resemble those seen with conventional nitrovasodilators. Doses $> D_T$ produce sustained (or *S*-type) responses, comprising an initial, rapid drop of pressure, followed by incomplete recovery, resulting in a plateau of reduced tone which can persist for several hours.

3 *T*- and *S*-type responses are attenuated by ferrohaemoglobin (Hb) and by methylene blue (MB), but not by inhibitors of endothelial NO· synthase. Addition of either Hb or MB to the internal perfusate can restore agonist-induced tone when administered during the plateau phase of an *S*-type response. Moreover, subsequent removal of Hb causes the artery to re-dilate fully.

4 We conclude that *T*- and *S*-type responses are both mediated by NO·. It is postulated that *S*-type responses represent the sum of two vasodilator components: a reversible component, superimposed upon a non-recoverable component. The former is attributed to free NO·, preformed in solution at the time of injection; and the latter to NO· generated by gradual decomposition of a 'store' of iron-sulphur-nitrosyl complexes within the tissue. This hypothesis is supported by histochemical studies which show that both clusters accumulate in endothelial cells.

Keywords: Nitric oxide donors; iron-sulphur-nitrosyls; vasodilator responses; nitrovasodilators; endothelium

Introduction

Endothelial cells release a labile factor which relaxes vascular smooth muscle (Furchgott & Zawadski, 1980). Endothelium-derived relaxing factor (or EDRF) has recently been identified as either nitric oxide (NO·; Palmer *et al.*, 1987; Ignarro *et al.*, 1988); a labile nitrosothiol, possibly S-nitroso-cysteine (Myers *et al.*, 1990); or a nitrosyl-iron complex with thiol ligands (Vanin, 1991). NO· is formed from the terminal guanidino nitrogen atom of L-arginine by a citrulline-forming enzyme, referred to as NO synthase (Palmer *et al.*, 1988a,b). Endothelial NO synthase (NOS) is NADPH- (Palmer & Moncada, 1989) and calmodulin-dependent (Bredt & Snyder, 1990). It can be inhibited by several L- (but not D-) analogues of arginine (Rees *et al.*, 1990).

Haemodynamic studies using stereospecific NOS inhibitors have firmly established the importance of NO· in controlling peripheral resistance *in vivo* (Vallance *et al.*, 1989; Aisaka *et al.*, 1989; Rees *et al.*, 1989; Gardiner *et al.*, 1990; Chu *et al.*, 1991). Endothelium-dependent relaxations of vascular smooth muscle are attenuated in some diseased states, notably in hypertension (Winquist *et al.*, 1984; Luscher & Vanhoutte, 1986; Otsuka *et al.*, 1988; Tesfamariam & Halpern, 1988; Sunano *et al.*, 1989) and in atherosclerosis (Harrison *et al.*, 1987; Chappell *et al.*, 1987; Henry *et al.*, 1987; Forstermann *et al.*, 1988, Guerra *et al.*, 1989), though it remains unclear whether these conditions are necessarily associated with impaired synthesis and/or release of NO· (Van der Voorde & Leusen, 1986; Hoeffner & Vanhoutte, 1989; Chester *et al.*, 1990; Jacobs *et al.*, 1990; Fozard & Part, 1991).

Clinical 'nitrovasodilators', including amyl nitrite, glyceryl trinitrate, nitroprusside (NP) and molsidomine, act independently of the endothelium, augmenting its function temporarily by releasing NO· *in vivo* (Waldman & Murad, 1984; Feelisch & Noak, 1987). Their hypotensive actions are generally of short duration and continuous drug infusions are necessary to effect sustained responses (Kreye, 1980). Here we show that two tetrairon-sulphur cluster nitrosyls (compound A: tetranitrosyl-tetra-μ3-sulphidotetrahedro-tetrairon; and compound B: heptanitrosyl-tri-μ3-thioxotetraferrate(1-), also known as Roussin's Black Salt; Roussin, 1858) are able to generate unusually protracted vasodilator responses when tested on rat isolated, internally-perfused tail artery preparations. The structures of both clusters, based on X-ray diffraction studies (Chu & Dahl, 1977; Chu *et al.*, 1982), are shown in Figure 1a,b. The vasodilator actions of A and B are blocked by haemoglobin and also by methylene blue, but not by agents which suppress NOS. Histochemical studies reveal that both clusters are able to penetrate cell membranes with extraordinary ease and that they accumulate inside the endothelium, forming a molecular 'store' of NO·. Our results suggest that their gradual decomposition from sites within the endothelium generates free NO· and that its slow release serves to sustain their vasodilator actions.

Preliminary accounts of some aspects of this work have appeared elsewhere (Flitney *et al.*, 1990; 1992)

Methods

Preparation

Experiments were performed on segments of tail artery taken from normotensive adult Wistar rats (250–460 g). Animals

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were killed by cervical dislocation. A length of artery (0.8–1.5 cm) was dissected free, cannulated (Portex cannula) and transferred to a Perspex bath.

Apparatus

The apparatus is shown in Figure 1. The cannula (C) formed part of a constant flow perfusion circuit, driven by a peristaltic pump (P1; Gilson Minipuls). The vessel (V) was perfused internally (flow rate: 2 ml min^{-1}) with solution pre-warmed by passage through a heat exchanger. Drugs were introduced into the lumen of the artery by bolus injection through a side tube (I). The outer surface of the vessel was superfused continuously with solution driven by a second peristaltic pump (P2). The temperature of the chamber was held at 30–35°C by adjustment of the flow rate in the external circuit (ca. 8 ml min^{-1}).

Light from an Argon ion laser (L; Spectra Physics Ltd., type 168-09) could be made to irradiate the artery directly, so as to induce photorelaxation of vascular smooth muscle (Furchgott *et al.*, 1961). The beam ($\lambda = 514.5$ nm; output intensity 1 mW) was reflected onto the preparation by means of a front-silvered mirror (M; beam diam. at preparation: approx 2 cm).

A differential pressure transducer (T: Sensym type SCX 150NC; Farnell Electronic Components, Leeds) detected changes in back pressure due to changes in arterial tone. Responses were displayed on a chart recorder.

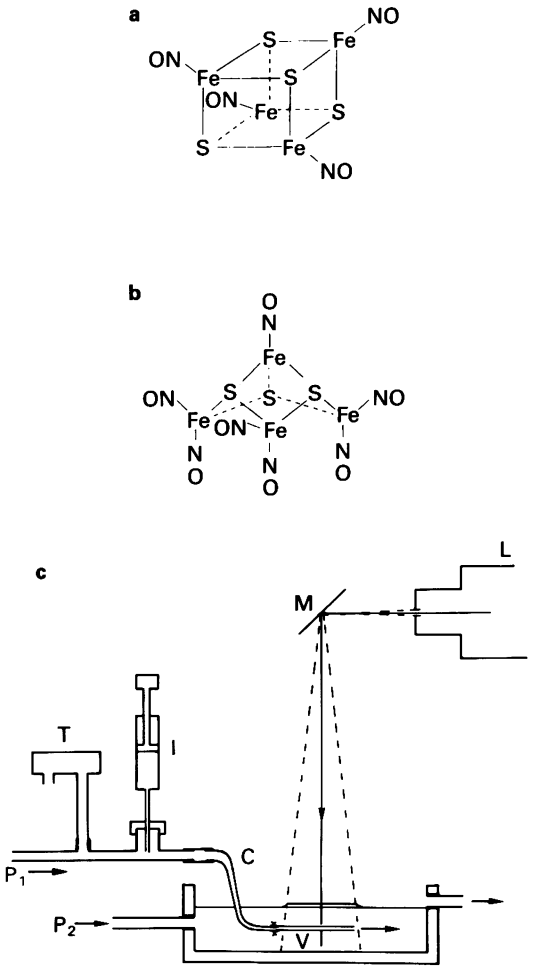


Figure 1 (a, b) Molecular structures of clusters A and B, as determined by X-ray diffraction studies. Compound A contains four and B seven ligated NO· groups per molecule. (c) Apparatus used for perfusing isolated segments of rat tail artery and for irradiating preparations with laser light. See text for full description and explanation of lettering.

Experimental protocol

Arteries were perfused internally with oxygenated Krebs solution (composition, mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with 95% O_2/5% CO_2 to maintain pH 7.4), initially at a low flow rate which was increased gradually over the next 10–20 min to reach a final value of 2 ml min^{-1}. The preparation was allowed to stabilize for 20–30 min, after which it was precontracted with Krebs plus phenylephrine HCl (= Krebs-+PE: mean (± s.e.) [PE] = 6.5 ± 0.5 μM. Mean (± s.e.) agonist-induced perfusion pressure = 101 ± 3.5mmHg).

Experiments were made to establish the mode of action of iron-sulphur cluster nitrosyls. Responses to bolus injections (10 μl) of A and B were compared with those evoked by (i) carbachol (CCh), an endothelium-dependent vasodilator; and (ii) either nitroprusside (NP), S-nitroso-N-acetylpenicillamine (SNAP) or exposure to laser light (L) all of which relax vascular smooth muscle independently of the endothelium.

The effects of adding the following to the internal perfusate were examined: (i) ferro-haemoglobin (Hb) a nitric oxide 'scavenger'; (ii) methylene blue (MB); and (iii) either N^G-monomethyl-L-arginine (L-NMMA) or N-nitro-L-arginine methyl ester (L-NAME), both stereospecific inhibitors of NOS. Comparisons were made with responses evoked by NP or SNAP.

Drugs: commercial sources and synthetic procedures

L-phenylephrine HCl (mol. wt. 203.7; Sigma Chemicals) was used at concentrations ranging from $2–12 \times 10^{-6}$M. Stock solutions (10^{-2}M) of NP (mol. wt. 298; BDH Ltd., 'Analar' grade) were made and the dose required adjusted by serial dilution immediately prior to use. SNAP (mol. wt. 220) was synthesized by reacting N-acetylpenicillamine with sodium nitrite (Field *et al.*, 1978). Compound B was prepared by reacting iron(II) sulphate heptahydrate, sodium nitrite and sodium sulphide in hot aqeous solution under nitrogen gas (Brauer, 1960). Compound A was prepared by addition of elemental sulphur to a solution of B dissolved in re-distilled toluene and refluxed overnight (16 h) under dry nitrogen gas (Gall *et al.*, 1974).

Purities of iron sulphur nitrosyl compounds were checked by (a) infra red spectroscopy (A had a single IR absorption maximum at wave number 1790 cm^{-1}; B had three maxima, at 1795 cm^{-1}, 1747 cm^{-1} and at 1707 cm^{-1}) and (b) ^{14}N-nuclear magnetic resonance (NMR) spectroscopy.

Solutions of B (mol. wt. 553) were made up in Krebs solution, immediately before use. Compound A (mol. wt. 472) is not soluble in water and so working solutions were made by serial dilution of 5 mg A dissolved in 1 ml dimethyl sulphoxide (ca. 10^{-2}M).

An important note concerning experimental procedures

All experiments were conducted in a darkened laboratory with a red safelight (60W) as the sole means of illumination. There were two reasons for this. First, the ambient lighting under normal laboratory conditions is sufficient to induce photorelaxation of precontracted vascular smooth muscle, causing a progressive loss of vessel tone. Second, NP, A and B are photosensitive and decompose when exposed to light, releasing free NO· (Wolfe & Swinehart, 1975; Flitney & Kennovin, 1988; Flitney *et al.*, unpublished results). Drug solutions were therefore made up in the dark and stored in glass vials covered with aluminium foil prior to use.

Results

Bolus injections of either NP or SNAP invariably produce fully-reversible (transient or *T*-type) vasodilator responses: an

initial, rapid drop of pressure, followed by complete recovery, often with some positive 'overshoot'. Compounds A and B produced *T*-type responses only at doses below a critical threshold concentration (D_T). Doses$>D_T$ generate long-lasting (sustained or *S*-type) responses, comprising an initial, rapid decrease of pressure, followed either by partial recovery only, or no recovery at all. The hallmark of the *S*-type response is a remarkably stable plateau of reduced vessel tone which can persist for several hours.

Mediation of T-*type responses by NO·*

Figure 2 compares vasodilator responses to carbachol (CCh), nitroprusside (NP) and laser light (L; = 514.5 nm; 1 mW intensity) with *T*-type responses produced by A and B. Control responses are shown in Figure 2a. The responses to A and B were blocked by either methylene blue (b; 75 μM) or by ferro-haemoglobin (c; 15 μM), a NO· scavenger. However, neither L-NMMA (d; 100 μM) nor L-NAME (not illustrated), both of which suppress endothelial NOS (Rees *et al.*, 1990), inhibited responses to A or B. Indeed, it was usual to find that treatment with either L-NMMA or L-NAME increased the responsiveness of arteries to A and B.

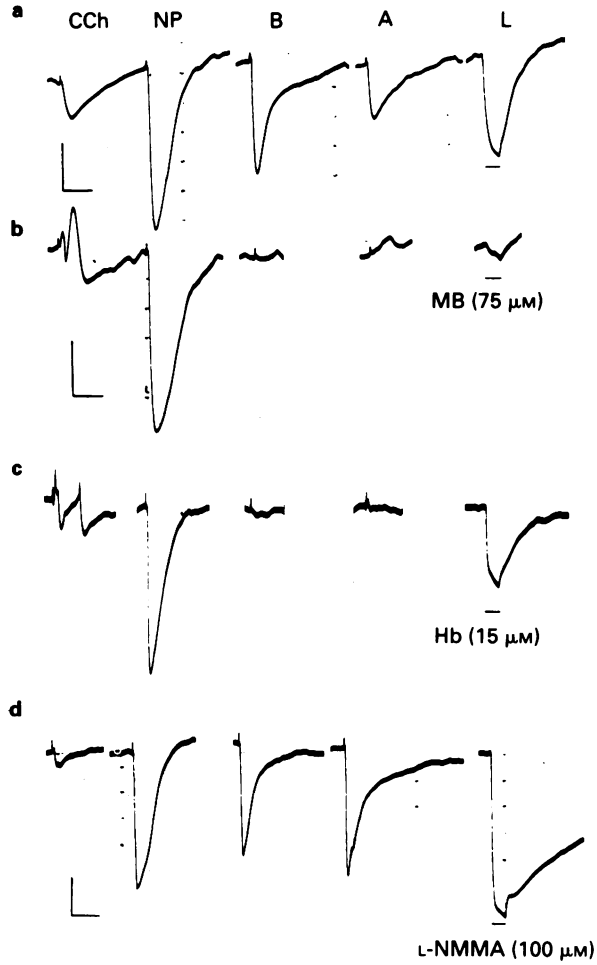


Figure 2 (a) Vasodilator responses produced by microinjection (10 μl) of test doses of carbachol (CCh: 10^{-2} M) and nitroprusside (NP; 2.5×10^{-4} M) and by irradiation with laser light (L; λ = 514.5 nm; 1 mW; 2 min), compared with *T*-type responses produced by clusters A (10^{-4} M) and B (5×10^{-4} M). Artery pre-contracted with 5×10^{-6} M phenylephrine (agonist-induced pressure = 135 mmHg). Female rat, 274 g. Temp., 32°C. (b) Suppression of responses to A and B and to laser light by treatment with methylene blue (MB 7.5×10^{-5} M). Details as above. (c) Inhibition of responses to compound A and B by haemoglobin (Hb, 15×10^{-6} M). Hb increased agonist-induced pressure to 1.8x control level (after ca. 6 min). Artery pre-contracted with 4×10^{-6} M phenylephrine (agonist-induced pressure = 114 mmHg). Female rat, 373 g. Temp. 32°C. (d) The NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 1×10^{-4} M) increased agonist-induced tone to around 1.28x the control level. Drugs tested 20 min after beginning perfusion with L-NMMA. Responses to carbachol attenuated by L-NMMA, but not those produced by nitroprusside (NP), A, B or laser light. Details as for (c) above. Calibration bars (all recordings): vertical, 20% agonist-induced tone (0.2Y; see Figure 3a); horizontal, 4 min.

S-*type responses produced by sequential injections of increasing doses of A or B*

Pressure recordings made by injection of increasing doses of NP (Figure 3c) or SNAP (not illustrated) confirm that both compounds produce *T*-type responses only. Similar experiments with A or B show that *T*-type responses give way to *S*-type responses when the injected dose exceeds D_T: both the rate and extent of the recovery following successive injections become progressively reduced, resulting in a stepwise and persistent loss of vessel tone (Figure 3a,b).

Three parameters have been measured (X, Y and Z; see Figure 3a) to enable quantitative comparisons to be drawn between NP and SNAP and either A or B. Figure 4 shows perfusion pressure minima (filled circles) immediately following injection of each drug (X) and the steady-state pressures attained after recovery (Z; open circles), both expressed as a fraction of the initial agonist-induced pressure (Y) and plotted as a function of $\log_{10}$ injected dose. The concentrations of SNAP, NP, A and B (μM) required to produce half-maximal

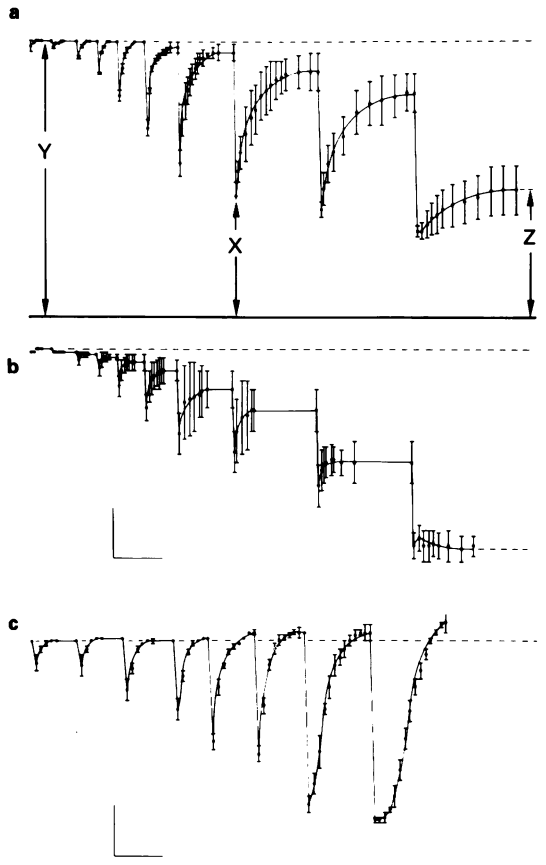
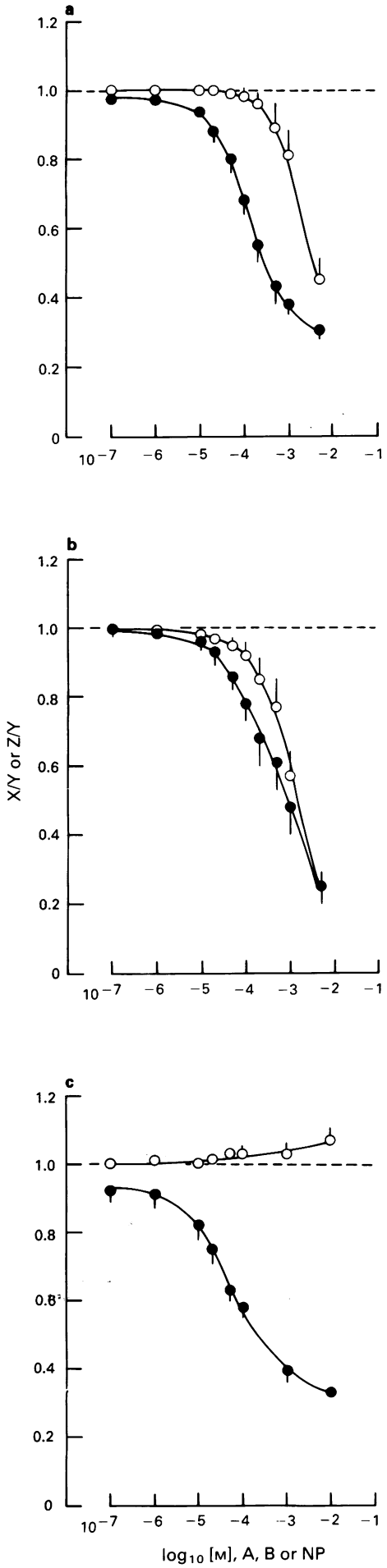


Figure 3 Averaged pressure recordings obtained by successive injections of increasing doses of compound A, B or nitroprusside (NP) (graphs a, b and c, respectively). Mean values (± s.e., vertical bars) are plotted ($n = 6$ preparations for a, and $n = 5$ for both b and c). Note that injection doses of A or B which exceed D_T (see text) produce a sustained drop in vessel tone (*S*-type response), whereas responses to NP are fully reversible (*T*-type response). In (a), Y = agonist-induced pressure; X = pressure minimum following each injection; and Z = steady-state (plateau) pressure attained after recovery (see also Figure 4). Calibration bars: vertical = 0.2Y; horizontal = 20 min (A and B) or 10 min (C).



decreases of X/Y (EC_{50} values) are 36, 43, 126 and 399, respectively. The corresponding curves for Z/Y reflect the tendency for A and B (but not NP) to generate S-type responses. The effect is more pronounced for B than for A, as shown by the greater proximity of the X/Y and Z/Y curves. D_T is estimated to be around 1–10 μM for B and 20–100 μM for A.

Abolition of the 'plateau' phase of the S*-type response by Hb or by MB*

The *S*-type response was remarkable in that even prolonged perfusion ('wash-out') with Krebs+PE solution alone (up to 5 h) failed to induce any further vasoconstriction, once the plateau phase was established. However, the addition of either Hb (15 μM) or MB (>10 μM; not illustrated) to the internal perfusate initiated a prompt and complete restoration of all agonist-induced tone; indeed, as Figure 5 clearly shows, the perfusion pressure rises well above the control

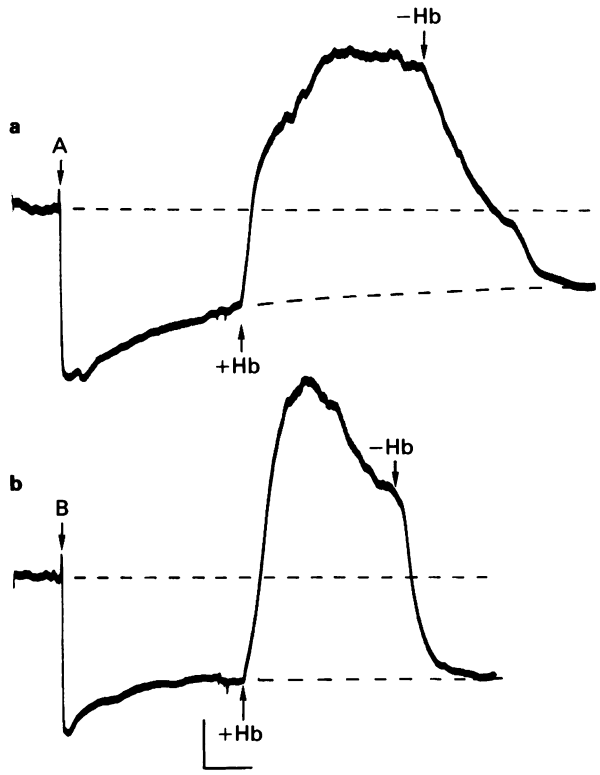


Figure 5(a, b) Pressure recordings from two preparations showing responses to ferro-haemoglobin (Hb, 31.5×10^{-5} M) added to, and then later removed from, the internal perfusate during the plateau phase of an *S*-type response. *S*-type responses were produced by a single bolus injection of 5×10^{-3} M A or B (upper and lower recordings respectively). The addition of Hb (upward arrows) elicits a prompt vasoconstriction which causes the perfusion pressure to rise above the control (pre-injection) value. Removal of Hb produces a vasodilatation which returns the pressure to the plateau value (curved dashed lines). Similar responses to these have been recorded when the time delay between the injection of A or B and treatment with Hb was increased to 6 h. Calibration bars: vertical = 0.2Y; horizontal = 4 min.

Figure 4 Mean (+ or − s.e., vertical lines values for X/Y (●) and Z/Y (○; see Figure 3a) plotted as a function of $\log_{10}$ injected dose for A (a), B (b) and nitroprusside (NP) (c). Responses to NP show complete recovery, with some pressure overshoot at the higher doses (Z/Y > 1.0). S-nitroso-N-acetylpenicillamine (SNAP) (data not shown) gives results which are qualitatively similar to those seen with NP. The values of Z/Y for both clusters (measured 20 min after each injection) are <1.0 at doses which exceed D_T (by definition; see text), reflecting the tendency for A and B to evoke *S*-type responses. Data obtained from same pressure recordings used to construct Figure 3 (a-c).

level (Y), here to around 1.6Y (A) and 1.8Y (B). Even more striking, the subsequent removal of Hb (but not of MB) causes arteries to re-dilate fully (Figure 5a,b). These observations lead us to conclude that extremely brief exposure of arteries to A or B (the 'transit' time for a 10 µl bolus passing through the vessel is ca. 0.3 s) can suffice to establish a durable source of NO· within the tissue.

Histochemical studies showing that iron sulphur cluster nitrosyls penetrate endothelial cells and accumulate there

The existence of a long-lasting source (store) of NO· in vessels exposed to either cluster is supported by microscopic studies of treated arteries. Solutions of A and B are intensely black and visual inspection of segments of artery subjected to repeated bolus injections (experiments of the type used to construct Figure 3) revealed some discolouration of the vessel lining. Microscopic examination of freshly-frozen, unfixed sections of artery exposed to cluster B (5 x 10^{-3}M, 5 min continuous perfusion, followed by 15 min washout with Krebs+PE to remove excess drug from the lumen of the artery) showed that the discolouration was confined to the endothelium (Figure 6a). Unfixed, frozen sections which had been stained with either bathophenanthroline (Figure 6b) or ferrocyanide (not shown), to detect iron(II) derived from the clusters (Pearse, 1972), showed a similar distribution of reaction product, located predominantly in endothelial cells, but sometimes with some faint staining of adjacent smooth muscle cells also. Control (untreated) arteries did not react positively for iron.

Discussion

This paper establishes iron-sulphur cluster nitrosyls as a novel class of NO· donor drug with unusual vasodilator properties. The compounds investigated here are able to generate two distinct kinds of response, designated *T*-and *S*-type responses. In this respect they differ from either NP or SNAP, both of which give fully-reversible responses only (Figures 3 and 4). The ability of Hb and of MB, but not NOS inhibitors, to block *T*- and *S*-type responses (Figure 2) shows that they are mediated by NO· derived from each cluster, and not by enhanced EDRF production through stimulation of the endothelial L-arginine:NO· pathway.

Composite nature of the S-type response

Our results lead us to postulate that the S-type response represents the sum of two, distinct vasodilator components: a reversible component, attributable to free NO· generated by the spontaneous decomposition of clusters in solution and present at the time of injection; superimposed on a 'non-recoverable' component, caused by the delayed release of NO· from clusters which have become trapped within the endothelium. This hypothesis is supported by histochemical studies (Figure 6), which revealed the presence of Fe(II) within endothelial cells of treated (but not control) arteries, and by our observation that the plateau phase of the *S*-type response can be abolished in a reversible manner by perfusing preparations with Hb (Figure 5).

Vanin and co-workers (Vanin, 1991) have shown that iron-dinitrosyls ($Fe(NO)_2$) complexed with low molecular weight thiols (e.g. cysteine or glutathione) can vasodilate isolated blood vessels and also inhibit platelet aggregation. Interestingly, the vasodilator action exhibits two-phase kinetics when tested on intact animals, resembling the *S*-type response described in the present study: that is, an initial, rapid decrease of arterial pressure, followed by a persistent (several hours) hypotensive action. The rapid and sustained components of the response are attributed to NO· released from (i) iron-dinitrosyl low-molecular thiol complexes (= rapid phase) and

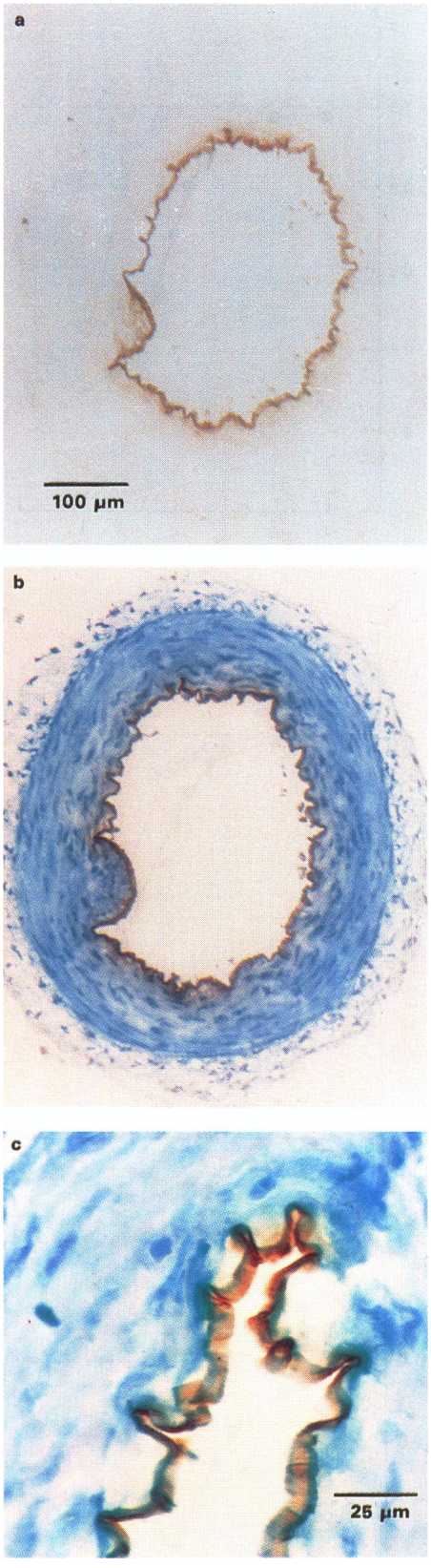


Figure 6 Transverse sections (20 µm) of a freshly-frozen artery perfused with B (5 mM, 2 ml min^{-1}) for 5 min, followed by 15 min perfusion (wash-out) with Krebs+PE solution only. (a) Unstained, unfixed section showing discolouration of the vessel lining. (b) Freshly-frozen, unfixed section after staining for 7 h with bathophenanthroline, followed by brief (4 min) counterstaining with methylene blue (scale as in (a)). Endothelial cells stain positively (red-brown colouration) for iron(ii) derived from the cluster. (c) Unfixed section stained for 4 min with methylene blue to show up smooth muscle cells. Note that brown colouration is confined to the endothelial lining of the vessel.

(ii) from iron-dinitrosyl protein-thiol complexes (= sustained phase) formed within the tissue. The latter are thought to result from the transfer of $Fe(NO)_2$ groups from low molecular weight to protein-borne ligands, forming a long-lasting, molecular 'store' of NO· in the vascular bed.

Suppression of endogenous EDRF production did not occur during the plateau phase of S-*type responses*

The effects of Hb administered during established *S*-type responses lead us to conclude that the formation of endogenous NO· from L-arginine is not suppressed by either cluster and that its continued release from endothelial cells helps to sustain the plateau phase. Thus, perfusing vessels with Hb does not merely re-establish the control (pre-injection) perfusion pressure, but instead drives it to a level substantially greater than this (Figure 5). The inference to be drawn is that Hb scavenges NO· from both exogenous (cluster-derived) and endothelial sources.

The nature of the intracellular mechanism(s) underlying the vasodilator actions of iron-sulphur nitrosyls remains to be established before meaningful comparisons can be made with other nitrovasodilators. The fact that Hb and MB can block responses to either cluster (and also to SNAP) but not those evoked by NP (Figure 2) clearly implies different cellular mechanisms of action. This has not been investigated in the present study. However, there is evidence to suggest that NP may relax vascular smooth muscle through both guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent and cyclic GMP-independent pathways (Otsuka *et al.*, 1988). A component of the relaxant effect of glyceryltrinitrate may also be mediated by a mechanism which does not involve elevated cyclic GMP levels (Diamond & Chu, 1983).

Physicochemical properties of iron-sulphur cluster nitrosyls

The physicochemical properties of iron sulphur cluster nitrosyls (Butler *et al.*, 1988) provide some insight into their vasodilator actions. Both compounds are potentially able to transport large quantities of NO·, with 4 and 7 mol. of ligated NO· per mol. of A and B, respectively. Their selective retention and gradual decomposition from within the endothelium can evidently generate physiologically significant amounts of NO· at this most relevant of sites. The mechanism by which NO· is released from clusters trapped inside the endothelium (whether spontaneous or enzymatic) is not known. Extended Hückel molecular orbital calculations show that A and B are electron precise (Sung *et al.*, 1985): this means that addition of an electron to A, and either the addition or removal of an electron in the case of B, will weaken the cage bonding and cause the iron sulphur framework to disintegrate, releasing free NO·. The oxidative status of the endothelial cell may therefore prove to be an important factor in determining the rate of NO· release *in vivo*.

The apparent ease with which both clusters are able to penetrate the endothelial cell membrane is probably related to their high solubility in non-polar solvents (Butler *et al.*, 1988). Compound B is especially interesting in this regard because it is ionic (A is neutral) and therefore soluble in polar solvents too. Curiously, though, the marked contrast in the intensity of staining as seen between endothelial cells and overlying smooth muscle cells (see Figure 6) shows that neither compound can traverse the endothelial cell layer readily. The faint reaction product which is sometimes discernible in smooth muscle cells immediately adjacent to the endothelium is probably a diffusion artefact of the staining procedure, because neither cluster survives conventional fixation methods (including vapour fixation) and so histochemical observations were of necessity made on freshly-frozen sections. This explanation is supported by preliminary X-ray microprobe studies of rapidly-quenched arteries following treatment with A or B, which reveal elemental Fe and S within endothelial cells, but no evidence for either in nearby smooth muscle cells (unpublished observations: Elder, Pediani, Megson & Flitney).

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